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Gender Differences in Circulating Levels of Neutrophil Extracellular Traps in Serum of Multiple Sclerosis Patients

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Abstract (100 words)

Neutrophil extracellular traps (NETs) trap and kill pathogens very efficiently but also activate dendritic cells and prime T cells. Previously, we demonstrated that neutrophils are primed and circulating NETs are elevated in relapsing remitting multiple sclerosis (RRMS), a T cell-mediated autoimmune disease. Here, we demonstrate gender specific differences in circulating NETs but not in neutrophil priming in RRMS patients. Although the results from our systematic and in depth characterization of these patients argue against a major role of circulating NETs in this disease, they suggest that NETs may underlie gender-specific differences in MS pathogenesis.

Key words: Neutrophil, neutrophil extracellular traps (NETs), multiple sclerosis, gender

1-Introduction

The main function of the innate immune system during infection is to eliminate the pathogen, and neutrophils are key players in innate immune responses. Women of reproductive age are more resistant to sepsis and subsequent morbidity and mortality than men (Schroder et al., 1998), and the incidence of sepsis in postmenopausal women increases to levels almost equal to those seen in age-matched men (Martin et al., 2003). Women also have a higher systemic neutrophil count compared with men (Bain and England, 1975a), and neutrophil counts correlate with estradiol levels during menstruation (Bain and England, 1975b) and pregnancy (Efrati et al., 1964) suggesting that sex hormones influence neutrophilia and overall resistance to sepsis most likely by delaying apoptosis in neutrophils (Molloy et al., 2003). In order to eliminate pathogens during infections, neutrophils are armed with a variety of weapons including engulfment and intracellular degradation of microbes (Hampton et al., 1998; Segal, 2005), release of oxygen species and granule proteins (Lehrer and Ganz, 1999) and release of extracellular chromatin fibers bound to granular, nuclear and cytoplasmic proteins called neutrophil extracellular traps (NETs) (Brinkmann et al., 2004). NETs not only trap and kill pathogens very efficiently but also minimize collateral tissue damage by containing proteases to the DNA fibers and act as physical barriers preventing microbial spread. Despite the well documented importance of NETs as an effective antimicrobial first line defense mechanism, there is increasing evidence that NETs occur in various clinical settings in the absence of microbial infections and that they are probably also associated with pathophysiological conditions (Amulic et al., 2012; Logters et al., 2009). NETosis, the process of neutrophil cell death that leads to expulsion of NETs, can be triggered by different stimuli including pro-inflammatory cytokines such as IL-8 and TNFα, phorbol 12-myristate 13-acetate (PMA) (Brinkmann et al., 2004; Fuchs et al., 2007), activated platelets (Clark et al., 2007) and endothelial cells (Gupta et al., 2010), and placental microparticles (Gupta et al., 2005). In addition to their bactericidal potential, NETs can also activate plasmacytoid- (pDCs) (Garcia-Romo et al., 2011; Lande et al., 2011) and myeloid (mDC) (Sangaletti et al., 2012) dendritic cells and in consequence modulate inflammatory responses.

The activation of DCs by NETs appears to play an important role in the pathogenesis of some autoimmune diseases such as psoriasis (Lande et al., 2007), systemic lupus erythematosus (SLE) (Barrat et al., 2005; Garcia-Romo et al., 2011; Lande et al., 2011; Means et al., 2005) small vessel vasculitis (Sangaletti et al., 2012) and type I diabetes (Diana et al., 2013). NETs-activated pDCs produce large amounts of interferon that can lead to the maturation of myeloid DCs (mDCs) and exert an effect on T cell function. In SLE, pathogenic auto-antibodies have been associated with abnormal clearing of NETs and NETosis that results in abnormally high production and/or low degradation of NETs, which then leads to tissue damage and facilitated generation of large quantities of auto-antibodies creating a vicious cycle (Knight and Kaplan, 2012). In small vessel vasculitis, NETs can favor neutropil proteins uploading into mDCs and the induction of anti-neutrophil cytoplasmic antibodies (ANCAs) (Sangaletti et al., 2012). Recently, we demonstrated that NETs are also able to directly prime T cells by reducing their activation threshold, which represents a novel link between innate and adaptive immune responses (Tillack et al., 2012).

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating disease of the central nervous system with aspects of secondary neurodegeneration (Sospedra and Martin, 2005). There are two major forms of MS, relapsing-remitting (RR)-MS, which affects around 85%-90% of patients, and primary progressive (PP)-MS in around 10%-15%. Most RR-MS patients later develop secondary progressive (SP)-MS. It is not clear at present, which factors are responsible for the different courses. RRMS affects women about twice as often as men, while no gender differences are observed in PPMS. The activation of $CD4^+$ autoreactive T cells and their differentiation into a T-helper type 1 (Th1) phenotype is a crucial event both in the initial phase and in the long-term evolution of RRMS. Damage of the central nervous system is however, most likely mediated by antibodies, complement, $CD8^+$ T cells, and factors produced by innate immune cells. Deregulation in other immune networks involving T-helper 2 (Th2) cells, regulatory $CD4^+$ T cells or NK cells are probably also involved. Compared to T cell- and antibody responses or to other innate immune cells such as DCs and monocytes, the role of neutrophils

in MS has not been examined extensively. In a previous study (Naegele et al., 2012), we demonstrated that neutrophils in RRMS patients are more numerous and exhibit a primed state. Furthermore we found higher levels of NETs in serum from these patients. Gender specific differences were not addressed in this study. The chronic inflammatory environment in RRMS (Lund et al., 2004) probably underlies this inappropriate neutrophil priming, which may result in enhanced neutrophil activation during infection. The higher levels of NETs in serum might also be linked to the chronic inflammatory environment in MS, but may have been caused by other triggers such as infections, that have been associated with relapses in these patients (Granieri et al., 2001) or higher frequency of activated platelets (Sheremata et al., 2008) or endothelial cells (Minagar et al., 2001) both elevated in MS. Due to the central role of T cells in RRMS and the ability of NETs to modulate T cell immunity, an abnormally gender-specific high level of NETs in this disease as consequence of neutrophil priming may play an important role in certain aspects of the pathogenesis and also explain some gender-specific differences.

Here, we have analyzed in detail a large cohort of MS patients including those with subforms other than RRMS, and we have found that only a subset of RRMS patients shows elevated circulating NETs. We also examined whether the higher levels of NETs as well as the primed state of neutrophils in these patients show sex differences, and our results indeed indicate that gender specific differences exist with respect to levels of circulating NETs but not in neutrophil priming. Further, we have performed a systematic and in depth characterization of these patients aimed to identify NETs triggers as well as their putative role in MS pathogenesis. The results argue against a major role of circulating NETs in MS but suggest that NETs may underlie gender-specific differences in MS pathogenesis.

2-Material and Methods

2.1 Patients

Patients were recruited from the inims outpatient clinic and day hospital at the University Medical Center Hamburg-Eppendorf. MS diagnosis was based on the revised McDonald criteria (Polman et al., 2005). Patients, who had not received steroids for at least 4 weeks prior to enrolment or any immunomodulatory or immunosuppressive agent during the last 3 months, were considered untreated and included in the study. Patients were classified into the following MS subgroups: patients with a first demyelinating event suggestive of MS, termed clinically isolated syndrome (CIS, n=126), RRMS (n=168) and PPMS (n=21). The CIS and RRMS group included patients that were either neurologically stable for at least 30 days before sampling (remission state, CIS n=76 and RRMS n=99) or exhibited an acute episode of neurological worsening lasting for more than 24 h at the time of sampling and not having received steroids (relapse state, CIS n=50 and RRMS n=69). Patients did not show clinical signs or symptoms of acute infection. Controls included patients with inflammatory neurological disease other than MS (OIND, n=5), patients with other non-inflammatory neurological diseases (OND, n=12) and a cohort of healthy volunteers not suffering from any known infectious or inflammatory disorder (HC, n=40). Table 1 summarizes the demographic characteristics of patients and controls. Supplementary Table 1 summarizes the diagnosis of patients with OIND and OND. This study was approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg), and written informed consent was obtained from all patients and controls before blood was drawn.

2.2 Quantification of circulating NETs and dsDNA in serum samples

Detection of myeloperoxidase (MPO) associated with DNA was used to quantify circulating NETs. MPO-DNA complexes in serum samples were quantified as previously described (Kessenbrock et al., 2009). Briefly, 5 µg/ml of mouse anti-human myeloperoxidase (MPO)-specific capture antibody (AbD SeroTec, Duesseldorf, Germany) was coated to 96-well plates. After blocking with 1% BSA, serum samples were added together with a peroxidase-labeled

anti-DNA monoclonal antibody (component 2 of the Cell Death ELISA kit, Roche). After 2 h of incubation, the peroxidase substrate was added according to manufacturer's instructions. Absorbance was measured at 405 nm using a µQuant microplate reader (Bio-Tek, Winooski, VT, USA). Total dsDNA in serum was quantified using Picogreen dsDNA kit (invitrogen, GIBCO, Paisley, UK) as previously described (Fuchs et al., 2007).

2.3 Neutrophil purification and in vitro stimulation

Neutrophils from RRMS patients and HC were isolated from freshly drawn peripheral blood using Dextran-Ficoll, as described previously (Weiss et al., 1985). Briefly, erythrocytes were sedimented for 30 minutes in Hank's balanced salt solution (HBSS) (Invitrogen GIBCO, Paisley, UK) containing 3% pure Dextran 200 (Serva, Heidelberg, Germany). The top phase was carefully layered onto 3 ml of LSM 1077 Ficoll solution (PAA Laboratories GmbH, Pasching, Austria) and centrifuged for 30 minutes at 2000 rpm without brakes. After centrifugation, the supernatant was discarded and the pellet washed. Remaining erythrocytes were lysed by adding 5 ml ice-cold H_2O for 20 sec. Neutrophil purity and viability was ≥97% and ≥95% respectively as assessed by expression of the neutrophil-specific marker CD16b and trypan blue exclusion as already described (Naegele et al., 2012).

Purified neutrophils were resuspended in HBSS (Invitrogen) medium supplemented with 10 mM Hepes (Invitrogen). 5 x 10^5-10^6 neutrophils/ml were seeded into tissue culture plates on glass coverslips (Menzel, GmbH, Braunschweig, Germany) pretreated with 0.001 % poly-L-lysine (Sigma-Aldrich, Steinheim, Germany). Neutrophils were stimulated with 25 nM PMA (Sigma-Aldrich) for 3 h.

2.4 Isolation and quantification of NETs

NETs released by activated neutrophils were digested with 10 U/ml micrococcal nuclease (MNase, Worthington Biochemical Corp. Lakewood, NJ) as previously described (Urban et al., 2009). NETs (MPO-DNA complexes) in supernatants were quantified using a capture ELISA as previously described (Kessenbrock et al., 2009). Absorbance was measured at 405 nm using a µQuant microplate reader (Bio-Tek, Winooski, VT, USA).

2.5 Protein- and cytokine determinations

C-reactive protein (CRP) and IL-6 levels in serum samples were measured at the central laboratory, University Medical Center Hamburg-Eppendorf. IL-8, platelet factor 4 (PF4), beta-thromboglobulin (beta-TG) and sVE-cadherin levels in serum samples were measured by ELISA using the following kits: Human IL-8 CytoSet™ (Biosource, Invitrogen), sCD144 (sVE-Cadherin) ELISA kit (PromoKine, PromoCell, GmbH, Germany), Asserumchrom® βTG (Diagnostica Stago, Parsippany, NJ) and RayBio® Human PF-4 ELISA kit (RayBiotech, Norcross, GA). Reactions were performed according to the manufacturer's instructions.

2.6 Blood cell counts

Peripheral blood was collected in EDTA-tubes and cell counts determined using an AcT Diff Coulter Counter (Beckman Coulter, Inc. Fullerton, CA) standardized with Coulter 4C-ES Cell Control (Beckman Coulter).

2.7 Intracellular cytokine staining

For intracellular cytokine staining, PBMCs were stimulated with PMA (50 ng/ml, Sigma) and ionomycin (1 μg/ml, Sigma) in the presence of Brefeldin A (10 μg/ml, eBioscience) for 5 h. Next, cells were stained with LIVE/DEAD® Fixable Dead Cell Stain Kit (AmCyan, Molecular Probes, Invitrogen), fixed and permeabilized with the corresponding buffers (eBioscience), and stained for CD3 (PE, DakoCytomation, Denmark), CD8 (PB, DakoCytomation, Denmark), IFNγ (FITC, BDPharmingen), IL-4 (PE-Cy7, eBioscience) and IL-17A (Alexa Fluor®-647, eBioscience) at room temperature. Sample acquisition was done with a LSR-II (BD) flow cytometer and data analyzed with FACS Diva (BD) and the FlowJo (Tree Star, Inc.) software packages.

2.8 Characterization of peripheral blood monocytes and dendritic cells

Human PBMCs were isolated from peripheral venous blood by density gradient and stained with anti-CD14 (Pacific Blue, Becton Dickinson, BD, Franklin Lakes, NJ), anti-CD16 (APC-Cy7, Biozol, Germany), anti-CD19 (Pacific Blue, BD) and anti-CD40 PE (PE, Miltenyi, Biotec, GmbH, Germany) for the characterization of monocytes and with anti-CD14, anti-CD19, anti-

CD40, anti-CD86 (FITC, Dako), anti-CD141 (APC, Miltenyi), anti-CD303 (APC, Miltenyi) and anti-CD1c (APC, Miltenyi) for the characterization of dendritic cells. Corresponding isotype controls were also measured. Sample acquisition was done with a LSR-II (BD) flow cytometer and data analyzed with FACS Diva (BD) and FlowJo (Tree Star, Inc.) software packages.

2.9 Autoantibodies determination

Anti-nuclear antibodies (ANAs), anti-neutrophil cytoplasmic antibodies (ANCAs) and anti-ds DNA titer in serum samples were determined using commercial assays (Euroimmun, Luebeck, Germany).

2.10 Statistical analysis

Statistical analyses were performed with Prism 5.02 (GraphPad Software Inc., San Diego, CA). Parametric tests were applied for two-group comparisons using unpaired t-tests with two-tailed p-values. Comparisons of three groups and more were assessed by one-way ANOVA with Bonferroni's correction for multiple comparisons. P-values < 0.05 were considered statistically significant.

3-Results

3.1 Gender differences in circulating levels of NETs in serum of RRMS patients

In order to explore whether higher levels of circulating NETs are a unique feature of RRMS patients, we quantified MPO-DNA complexes in serum of other MS and non-MS patients including CIS (n=126), PPMS (n=21), OIND (n=5) and OND (n=10) patients. Significant differences in the level of MPO-DNA complexes were observed in RRMS and OIND patients but not in CIS, PPMS or OND patients compared with HC (Fig. 1A). The amount of MPO-DNA complexes in serum of RRMS patients showed strong inter-individual variability, with optic density (OD) at 405 nm ranging from 0.107 to 2.288. In order to facilitate analysis, we have arbitrarily defined a threshold of OD > 0.3 to identify patients with high content of MPO-DNA complexes in serum. 17.7 % of RRMS and 60% of OIND patients showed OD > 0.3, i.e. high levels of MPO-DNA complexes in serum. The percentage of HC (7.5 %), CIS (8.8 %), PPMS (4.7 %) or OND (10 %) patients belonging to this group was lower (Fig. 1A).

We also analyzed whether MPO-DNA complexes were present in CSF samples of CIS (n=20), RRMS (n=21), PPMS (6), OIND (5) and OND (10) patients. No MPO-DNA complexes were detectable in CSF (Fig. 1B).

MPO-DNA complexes did not correlate with age (Fig. 1C) but unexpectedly were significantly higher in males compared with females (Fig. 1D). When the different groups of patients and HC were analyzed independently, sex-specific differences were statistically significant only in RRMS patients. 12.1% of female RRMS patients showed OD > 0.3, i.e. high levels of MPO-DNA complexes in serum, compared to 26.2% of male RRMS patients (Fig. 1D). Interestingly, our previous observation that neutrophils in RRMS patients are more numerous and exhibit a primed state does not seem to underlie the gender-specific differences in circulating levels of MPO-DNA complexes, since neutrophil counts, neutrophil expression of fMLP receptor (FPR1), IL-8 receptor (CXCR1), TLR2, CD43 and CD63, all markers of neutrophil priming

and significantly higher in RRMS patients (Naegele et al., 2012), did not show gender-specific differences (Supplementary Fig. 1).

3.2 Gender differences in NETs formation and degradation

In order to explore whether NETosis can be more easily induced in male RRMS neutrophils, which could explain the gender-specific differences in MPO-DNA complexes in serum, we compared the ability of purified neutrophils from female and male RRMS patients and HC to release NETs in vitro. Purified neutrophils from female (n=20) and male (n=10) RRMS patients and female (n=33) and male (n=22) HC were stimulated in vitro with PMA and NETs were isolated and quantified using an MPO-DNA capture ELISA. Results are summarized in Figure 2A. No significant differences in NET formation were observed between neutrophils from female and male RRMS patients and HC, although females from both groups showed a tendency to release more MPO-DNA complexes than males.

Next, in order to examine whether gender differences in DNAse activity could be involved in the high level of MPO-DNA complexes in male RRMS patients, we have compared cell-free dsDNA in serum of pre-selected groups of female and male RRMS and CIS patients, each group containing a similar number of individuals with high (OD > 0.3, RRMS female n=9 and male n=9, CIS female n=7, males=7) and low (OD < 0.2, RRMS female n=9 and male n=9, CIS female n=6, males=8) levels of MPO-DNA complexes (Fig. 2B). No correlation between NETs and dsDNA was observed in these groups of patients, but interestingly, male RRMS- but not CIS patients showed a significantly higher level of cell free dsDNA in serum compared with females (Fig. 2B). When the level of dsDNA in female and male RRMS and CIS patients containing high or low levels of MPO-DNA complexes was compared (Fig. 2C), a significantly higher level of dsDNA was found in serum of male RRMS with high MPO-DNA complexes compared with females with low complexes, while no differences were found in CIS patients.

3.3 Circulating NETs in serum and disease activity

In order to characterize circulating levels of NETs in serum of RRMS patients, we measured MPO-DNA complexes in different serum samples obtained from

8 individual patients (4 females and 4 males) at different time points (Fig. 3A). We found that high levels of NETs (OD > 0.3) were not stable but fluctuated over time. In some patients the analyzed serum samples never reached an OD > 0.3, indicating low levels of circulating NETs. Interestingly, patient 2 showed a very high level of circulating NETs over a period of 30 months.

With the purpose to examine whether these fluctuations were related with disease activity, we compared serum samples of females and males CIS and RRMS patients in remission and relapse (CIS females n=40 remission and 38 relapse, CIS males n=29 remission and 19 relapse; RRMS females n=63 remission and 43 relapse, RRMS males n=36 remission and 26 relapse). No significant differences in the amounts of MPO-DNA complexes were observed between patients in remission and relapse (Fig. 3B). We also analyzed 22 RRMS patients, from whom paired serum samples corresponding to relapse and remission were available (Fig. 3C). In most patients higher or similar amounts of MPO-DNA complexes were detected in relapse while only in a few patients the amount was higher during remission.

3.4 Putative triggers of NET formation in RRMS patients

To address a putative link between NETs release and inflammation, we compared the level of serum IL-8 as an indicator of inflammation in pre-selected groups of female and male RRMS patients with either high (OD > 0.3, females n=9, males n=9) or low (OD < 0.2, females n=9, males n=9) levels of MPO-DNA complexes. As shown in Figure 4, no significant differences were observed between the different groups of patients. We also explored a putative role of infections in NETs release, by measuring C-reactive protein (CRP) and IL-6. CRP is an acute-phase protein that rises in response to inflammation, and IL-6 is also an important mediator of acute-phase responses. No significant differences in CRP or IL-6 levels between the different groups of patients were found (Fig. 4). Finally, we also examined a putative role of platelet and endothelial activation in NETosis by measuring the levels of platelet activation factor (PF4) and soluble beta-thromboglobulin (beta-TG) (Fig. 4) as markers of platelet activation (Kaplan and Owen, 1981; Stuckey et al., 1992) and soluble sVE cadherin (CD144, Fig. 4) as marker of endothelial activation and damage (Vestweber, 2008). No significant

differences in PAF, beta-TG or sVE cadherin levels between the different groups of patients were observed.

3.5 Circulating NETs and peripheral blood lymphocytes

Next, in order to explore a putative role of NETs in gender-specific differences in MS pathogenesis, we examined whether gender as well as the level of MPO-DNA complexes in serum have an effect on the circulating number of lymphocytes. First we compared the absolute lymphocyte counts in whole blood of females and males RRMS and HC (RRMS females n=35, males n=20; HC females n=25, males n=15), and no differences were found (Fig. 5A). Then we measured absolute lymphocyte counts in female and male RRMS patients with high (OD > 3; females n=12, males n=9) and low (OD < 0.2; females n=13, males n=10) MPO-DNA complexes in serum. Again no significant differences were found between the different groups (Fig. 5B). Circulating lymphocytes were further characterized by flow cytometry for a putative effect of gender and circulating MPO-DNA complexes on their functional phenotype. The percentages of $CD8^+$ IFNγ-producing cells as well as of $CD4^+$ Th1 (IFNγ-producing), Th2 (IL-4-producing) or Th17 (IL-17-producing) cells were measured by intracellular cytokine staining in PBMCs of female and male RRMS patients with high (OD > 3; females n=8, males n=8) and low (OD < 0.2; females n=8, males n=8) MPO-DNA complexes in serum. No differences in the percentage of any of these T cell populations were found between the different groups of patients (Fig. 5C and D).

3.6 Circulating NETs and peripheral blood dendritic cells

Since it has been demonstrated that NETs can activate DCs, we also examined whether gender and the level of MPO-DNA complexes in serum have an effect on circulating DCs. We measured by flow cytometry the percentages of plasmacytoid, myeloid type 1 and myeloid type 2 DCs as well as their expression of CD40 and CD86 in female and male RRMS patients with high (OD > 0.3; females n=15, males n=13) and low (OD < 0.2; females n=14, males n=13) MPO-DNA complexes in serum (Fig. 6). We did not find significant differences in the percentage of any of these DC populations nor in their activation state between the different groups of patients.

3.7 Circulating NETs and peripheral blood monocytes

Subsequently, we also examined whether gender and level of MPO-DNA complexes in serum have an effect on circulating monocytes. First, we compared the absolute monocyte counts in whole blood of females and males RRMS and HC (RRMS females n=35, males n=20; HC females n=25, males n=15), and no differences were found (Fig. 7A). Then we measured absolute monocyte counts in female and male RRMS patients with high (OD > 3; females n=12, males n=9) and low (OD < 0.2; females n=13, males n=10) MPO-DNA complexes in serum. Unexpectedly, significantly higher monocyte counts were found in RRMS patients with high MPO-DNA complexes in serum, and interestingly the strong difference was observed between males with high and females with low MPO-DNA complexes in serum (Fig. 7B). In order to better understand the putative effect of gender and NETs on monocyte expansion, we also measured by flow cytometry in PBMCs the percentage of classical, intermediate and non-classical monocytes as well their expression of CD40 in female and male RRMS patients with high (OD > 0.3; females n=10, males n=7) and low (OD < 0.2; females n=9, males n=7) MPO-DNA complexes in serum. We did not find significant differences in the percentage of any of these monocyte populations and neither regarding their activation state (Fig. 7C).

3.8 Autoantibodies in RRMS patients with NETs in serum

Finally, since pathogenic antinuclear (ANA), ANCA and anti-dsDNA auto-antibodies have been associated with abnormal NETs clearing and NETosis in SLE (Knight and Kaplan, 2012), anti-dsDNA auto-antibodies are more prevalent among male SLE patients, and further since ANCAs have been found elevated in patients with the optico-spinal form of MS in Japan (Fukazawa et al., 1996), we measured ANA, cANCA, pANCA and anti-dsDNA antibody levels in serum samples of female and male RRMS patients with high (OD > 3; females n=6, males n=5) and low (OD < 0.2; females n=6, males n=4) MPO-DNA complexes. No anti-dsDNA nor cANCA autoantibodies were detected in any sample and for ANA and cANCA, although a tendency to higher levels in males was observed, differences did not reach significance (Table 2).

4-Discussion

NETs are extracellular chromatin fibers bound to granular, nuclear and cytoplasmic proteins that despite their importance as effective antimicrobial first line defense mechanism, also occur in various clinical settings in the absence of microbial infections and might be associated with pathophysiological conditions (Logters et al., 2009). In a previous study (Naegele et al., 2012), we demonstrated that neutrophils in RRMS patients are more numerous and exhibit a primed state. Among the features of neutrophils in these patients, we found significantly higher levels of NETs in the serum and speculated that these may be involved in MS pathogenesis. To examine this issue in depth, we analyzed here a large cohort of MS patients including patients suffering from subforms of MS other than RRMS, and found that only a small subset of RRMS patients have elevated circulating NETs. In view of the fact that women of reproductive age have a higher systemic neutrophil count compared with men and that RRMS affects women about twice as often as men we then examined whether the level of NETs in serum as well as neutrophil counts and primed phenotype in RRMS patients were higher in females. Unexpectedly, our results demonstrate that the subset of RRMS patients with high NETs in serum was significantly enriched in male patients while no gender specific differences were observed in neutrophil counts and primed state. Although higher circulating NET levels do not seem to be a general feature of RRMS patients, they might still be relevant in the above subset of RRMS patients and underlie gender-specific differences in MS pathogenesis. MS is a highly heterogeneous disease most likely involving very diverse pathomechanisms. Neutrophils are normally not present in the CNS of MS patients, and consistent with this notion we did not detect NETs in CSF samples from these patients. Considering the potential dangers associated with the indiscriminate histotoxic potential of neutrophils there may be active mechanisms preventing their entry into the vulnerable CNS compartment. However, these cells are likely to play a role in opening the blood brain barrier, particularly via NETosis, and this potential mechanism deserves further investigation.

The higher circulating NETs in a subset of RRMS patients and particularly in males could reflect an alteration in NET formation and/or in NET degradation. Neutrophils in RRMS patients are primed and can easily be activated (Naegele et al., 2012), which could explain higher NETosis in RRMS patients. However, our observation that granulocyte counts as well as the neutrophil primed state did not show gender-specific differences argues against this hypothesis. To explore further this possibility, we also analyzed NETosis in vitro using purified neutrophils from female and male RRMS patients and HC. We did not find differences in NETosis between purified neutrophils from RRMS and HC and neither gender-specific significant differences. In contrast, male RRMS patients showed significantly higher cell-free dsDNA in serum compared with female patients suggesting a putative abnormal DNAse activity that might underlie the higher circulating NETs levels in male RRMS patients. Interestingly, in SLE, in which women are affected about 10 times more frequently than men, it is interesting to note that male gender has been associated with more severe disease and with higher prevalence of pathogenic anti-dsDNA autoantibodies (Freire de Carvalho et al., 2010; Lu et al., 2010; Molina et al., 1996). Similarly, RRMS affects women about twice as often as men, and overall male gender has also been associated with worse prognosis. Although some small studies reported that men with MS are prone to develop less inflammatory but more destructive lesions than women (Pozzilli et al., 2003), have less T2 and gadolinium(Gd)-enhancing lesions (Weatherby et al., 2000) but more T1 lesions (van Walderveen et al., 2001) and more brain atrophy (Antulov et al., 2009), gender-specific MRI differences have not been confirmed by large studies (Fazekas et al., 2009). However, gender appears to influence disability during the early phase of RRMS. When males are compared with females, they do not show differences at the age of RRMS onset, but females progress more slowly and thus are overall older when they reach a disability status scale of 3 (Leray et al., 2010). Since disability evolution in RRMS correlates with axonal injury and this in turn correlates with inflammation, all these data suggest that the nature of inflammation and/or a higher vulnerability of CNS tissue in RRMS males might underlie the worse prognosis in these patients. Higher circulating NETs levels

might prime T cells and other immune cell types as well as facilitate their migration through the blood brain barrier and, as a consequence, lead to more destructive neuroinflammatory processes in male RRMS patients.

In order to understand better the potential causes of NETosis in RRMS patients we have performed a systematic and in depth characterization of these patients. Our data show that high levels of NETs are not stable in serum, but fluctuate over time. While this might indicate an association with disease activity, which comes and goes in MS, we could not demonstrate a correlation between relapses and higher circulating NETs in serum. Regarding potential triggers, we could not either demonstrate an involvement of inflammation, infection, platelet nor endothelial activation. One important question to consider in the interpretation of these results is the stability and half-life of MPO/DNA complexes in serum. Different from free DNA that is easily digested by DNAses, MPO-DNA complexes appear to be much more stable, and consequently their presence may not always indicate recent NETosis. This open issue might be one of the reasons for our difficulties to identify triggers of NETs.

Further we have addressed a putative role of these complexes in RRMS pathogenesis and gender-specific differences by systematically characterizing circulating lymphocytes, DCs and monocytes. Unfortunately, the analysis of circulating lymphocytes and DCs did not provide information regarding a putative role of NETs in MS pathogenesis and neither regarding gender-specific differences. Unexpectedly, the analysis of circulating monocytes showed significant higher monocyte counts in whole blood of RRMS patients with higher circulating NETs and particularly in male patients. The phenotypic characterization of classical, intermediate and non-classical monocytes in these patients did not allow us to identify the monocyte population expanded, which suggests that we are either missing other monocyte populations or that our cell counter-based monocyte counts enumerate as monocytes another cell type with similar physical features. The identification and characterization of this cell population expanded in RRMS with high NETs requires further investigation.

Finally, we also examined a putative link between NETs in serum and autoantibody production in RRMS patients. ds-DNA auto-antibodies and cANCA were not detected in any sample. Regarding ANA and pANCA, although the differences were not significant, the tendency to higher level in males might deserve further investigation. As mentioned above with respect to triggers of NETs, the lack of information concerning the exact time point of NETosis most likely hinders the search for a putative role of these complexes in RRMS pathogenesis.

The well documented role of NETs in the pathogenesis of some autoimmune diseases, their ability to modulate T cell immunity and our previous observation that higher levels of NETs in serum characterize a subset of RRMS patients encouraged us to analyze in detail whether abnormally high levels of NETs may play a role in certain aspects of this T cell-mediated autoimmune disease. As discussed above, the low frequency of RRMS patients with high levels of circulating NETs as well as the results from our characterization of these patients argue against a major role of NETosis in disease pathogenesis. However, our observation that individuals with high level of circulating NETs were significantly higher among RRMS patients than among HC or patients with other MS subforms, and more importantly that the frequency of males belonging to this subset was significantly higher than of females, suggests that NETosis might be responsible for some of the gender-specific differences in this disease and be involved in some aspects of MS pathogenesis such as opening of the blood brain barrier. Further investigation of gender-specific differences in NETosis using other methodological approaches might clarify its role in MS pathomechanisms and -heterogeneity.

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Figure legends

Figure 1. Gender differences in circulating levels of NETs in serum of RRMS patients. A) Quantification of MPO-DNA complexes in serum samples of HC (n=40), CIS (n=126), RRMS (n=168), PPMS (n=21), OIND (n=5) and OND (n=10) patients. An arbitrary threshold of OD > 0.3 to differentiate samples with high MPO-DNA complexes is shown (dotted line). Percentage of donors/patients with OD > 0.3, the mean OD as measured by capture ELISA and the statistical significance are shown. (* $p<0.05$). **B)** Quantification of MPO-DNA complexes in CSF samples from CIS (n=20), RRMS (n=21), PPMS (n=6), OIND (n=5) and OND (n=10) patients. **C)** Correlation between age and MPO-DNA complexes in all serum samples. **D)** Quantification of MPO-DNA complexes in serum samples of all females (n=225) and males (n=149), of HC females (n=20) and males (n=20), of CIS females (n=78) and males (n=50), of RRMS females (n=107) and males (n=61) and of PPMS females (n=10) and males (n=9). Percentage of donors/patients with OD > 0.3, the mean optical density and statistical significance are shown. (* $p=0.0113$, ** $p=0.0058$).

Figure 2. Gender differences in NETs formation and degradation. A) Quantification of MPO-DNA complexes released in vitro by purified neutrophils from female (n=33) and male (n=22) HC and female (n=20) and male (n=8) RRMS patients after stimulation with 25 nM PMA. The mean stimulation index (SI) is shown. (SI= OD measured by capture ELISA in stimulated neutrophils / OD in unstimulated neutrophils. **B)** Quantification of MPO-DNA complexes in serum samples from pre-selected groups of female and male RRMS patients and CIS patients each group containing a equal number of individuals with high (OD > 0.3, RRMS female n=9 and male n=9, CIS female n=7, males=7) or low (OD < 0.2, RRMS female n=9 and male n=9, CIS female n=6, males=8) levels of MPO-DNA complexes (upper graphs). dsDNA (ng/ml) quantified using Picogreen in the same groups of female and male patients (lower graphs). Mean and statistical significance are shown. (** $p=0.0046$). **C)** dsDNA (ng/ml) quantified using Picogreen in the same groups of female and male patients with high (OD>0.3) and low

(OD<0.2) MPO DNA complexes. Mean and statistical significance are shown. (**p<0.01).

Figure 3. Circulating NETs in serum and disease activity. **A)** Quantification of MPO-DNA complexes in serum samples of 8 RRMS patients (4 females/4 males) obtained at different time points. **B)** Quantification of MPO-DNA complexes in serum samples of CIS females (n=40 remission and 38 relapse), CIS males (n=29 remission and 19 relapse), RRMS females (n=63 remission and 43 relapse) and RRMS males (n=36 remission and 26 relapse) **C)** Quantification of MPO-DNA complexes in serum samples from female (upper graphs) and male (lower graphs) RRMS patients for whose paired samples in remission and relapse were available. The left graphs shows patients with higher MPO-DNA complexes in relapse than in remission, while the right graph shows patients with higher MPO-DNA complexes in remission than in relapse. An arbitrary threshold of OD > 0.3 to differentiate samples with high MPO-DNA complexes is shown in all graphs (dotted line).

Figure 4. Putative triggers of NET formation in RRMS patients. A) IL-8, CRP, IL-6, PF4, beta-TG and sVE cadherin quantification in serum samples of pre-selected groups of female (upper graphs) and male (lower graphs) RRMS patients with high (OD > 0.3, female n=9, male n=9) and low (OD < 0.2, female n=9, male n=9) MPO-DNA complexes. The mean is shown in all graphs.

Figure 5. Circulating NETs and peripheral blood lymphocytes. A) Absolute peripheral blood counts of lymphocytes obtained using a coulter counter in female and male HC and RRMS patients (RRMS females n=35, males n=20; HC females n=25, males n=15). **B)** Absolute peripheral blood lymphocyte counts in female and male RRMS patients with high (OD > 3; females n=12, males n=9) and low (OD < 0.2; females n=13, males n=10) MPO-DNA complexes in serum. Mean ± SEM are shown. Frequency (%) determined by flow cytometry of $CD3^+CD8^+$ and $CD3^+CD8^+$ IFNγ-producing T cells (CD8+ IFNγ+) **(C),** and $CD3^+CD4^+$, Th1 ($CD3^+CD4^+$ $IFN\gamma^+$ $IL\text{-}4^-$ $IL\text{-}17A^-$), Th2 ($CD3^+CD4^+$ $IFN\gamma^-$ $IL\text{-}4^+$) and Th17 ($CD3^+CD4^+$ $IFN\gamma^-$ $IL\text{-}17A^+$) T cells **(D)** in PBMCs of female and male RRMS patients with high (OD > 0.3, female

n=8, male n=8) and low (OD < 0.2, female n=8, male n=8) MPO-DNA complexes in serum. Mean is shown in all graphs.

Figure 6. Circulating NETs and peripheral blood dendritic cells. Gating strategy used to identify mDCs type 1 ($CD1c^+$), mDCs type 2 ($CD141^+$) and pDCs ($CD303^+$) (upper dot plots). Frequency (%, upper graphs) of the different DCs and CD40 and CD86 expression (MedFI, lower graphs) in PBMCs of female and male RRMS patients with high (OD > 0.3; females n=15, males n=13) and low (OD < 0.2; females n=14, males n=13) MPO-DNA complexes in serum. Mean is shown in all graphs.

Figure 7. Circulating NETs and peripheral blood monocytes. A) Absolute peripheral blood counts of monocytes obtained using a coulter counter in female and male HC and RRMS patients (RRMS females n=35, males n=20; HC females n=25, males n=15). **B)** Absolute peripheral blood monocyte counts in female and male RRMS patients with high (OD > 3; females n=12, males n=9) and low (OD < 0.2; females n=13, males n=10) MPO-DNA complexes in serum. Mean ± SEM and statistical significance are shown. (*p=0.0124; ***p<0.001). **C)** Frequency (%, upper graphs) of classical, intermediate and non-classical monocytes and CD40 expression (MedFI, lower graphs) in PBMCs of female and male RRMS patients with high (OD > 0.3; females n=10, males n=7) and low (OD < 0.2; females n=9, males n=7) MPO-DNA complexes in serum. Mean is shown in all graphs.

Table 1. Demographic characteristics of MS patients and controls.

Group	n	F	M	F:M	Mean Age ± SD
CIS	126	78	48	1.6	33.9 ± 8.8
• CIS remission	76	48	28	1.7	35 ± 9.4
• CIS relapse	50	30	20	1.5	32.3 ± 7.6
RRMS	168	106	62	1.7	36.5 ± 7.9
• RRMS remission	99	63	36	1.7	37.1 ± 7.5
• RRMS relapse	69	43	26	1.6	35.7 ± 8.5
PPMS	21	11	10	1.1	39.3 ± 6.6
OIND	5	3	2	1.5	47.8 ± 20.5
OND	10	5	5	1	45.3 ± 11
HC	40	20	20	1	32.2 ± 7.3

Table 2. Autoantibodies in female and male RRMS patients with low and high MPO-DNA complexes in serum

GENDER	OD (MPO-DNA)	ANA	cANCA	pANCA	Anti-dsDNA
FEMALES					
	0,12	neg	neg	neg	neg
	0,125	neg	neg	neg	neg
	0,187	pos (1/320)	neg	neg	neg
	0,11	pos (1/1000)	neg	neg	neg
	0,126	neg	neg	neg	neg
	0,135	neg	neg	neg	neg
MALES					
	0,121	pos (1/320)	neg	pos (IgG 1/10)	neg
	0,131	neg	neg	neg	neg
	0,155	pos (1/1000)	neg	pos (IgG 1/10)	neg
	0,21	pos (1/1000)	neg	neg	neg
FEMALES					
	0,413	neg	neg	neg	neg
	0,477	neg	neg	neg	neg
	0,779	neg	neg	neg	neg
	0,901	neg	neg	neg	neg
	1,291	neg	neg	pos (IgG 1/10)	neg
	1,439	neg	neg	neg	neg
MALES					
	0,451	neg	neg	neg	neg
	0,491	neg	neg	neg	neg
	0,569	pos (1/320)	neg	neg	neg
	0,782	pos (1/320)	neg	neg	neg
	2,143	neg	neg	pos (IgG 1/10)	neg

Figure 1

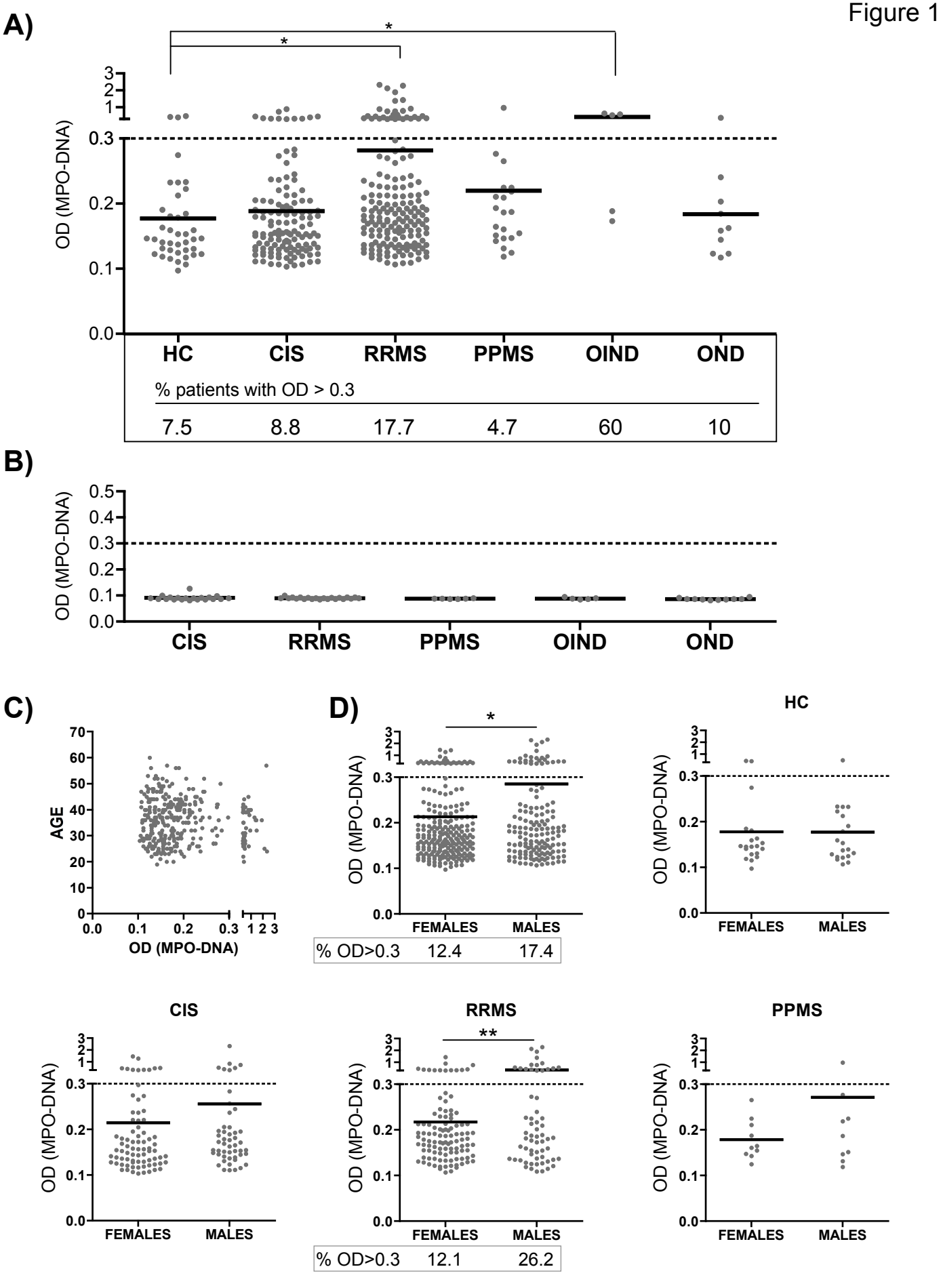

A)
*
*
OD (MPO-DNA)
HC
CIS
RRMS
PPMS
OIND
OND
% patients with OD > 0.3
7.5
8.8
17.7
4.7
60
10
B)
OD (MPO-DNA)
CIS
RRMS
PPMS
OIND
OND
C)
AGE
OD (MPO-DNA)
D)
*
OD (MPO-DNA)
FEMALES
MALES
% OD>0.3
12.4
17.4
HC
CIS
RRMS
**
PPMS
% OD>0.3
12.1
26.2

Figure 2

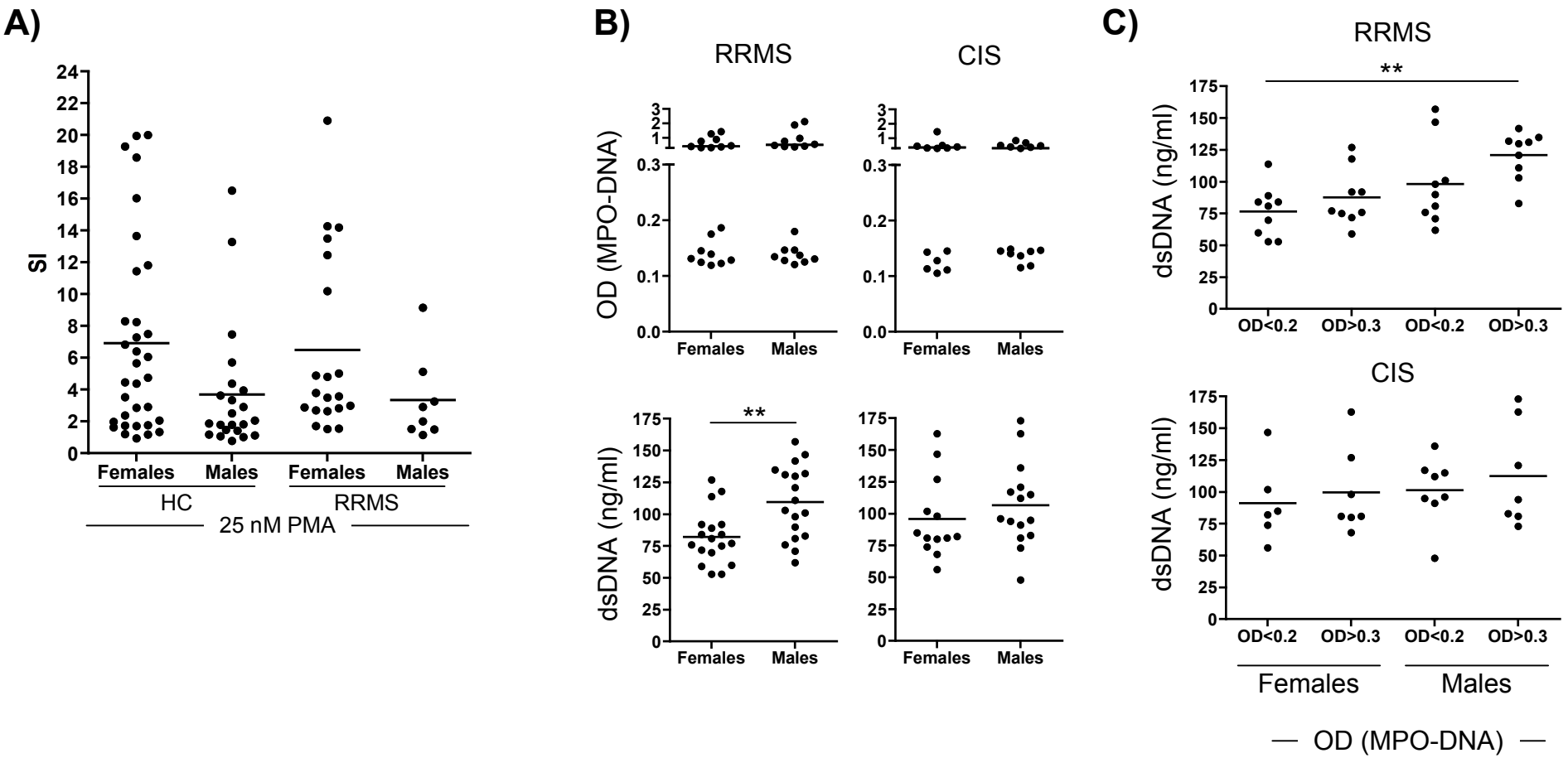
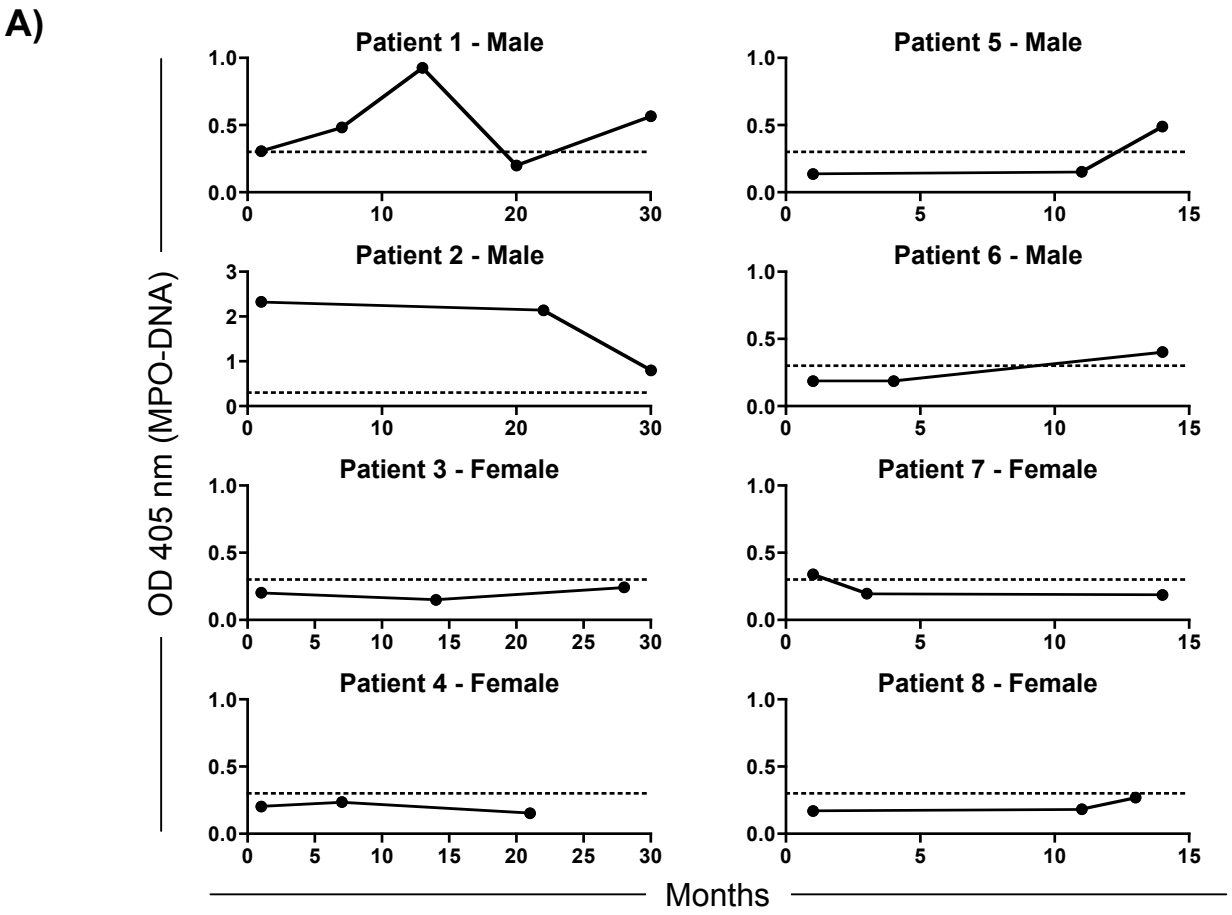
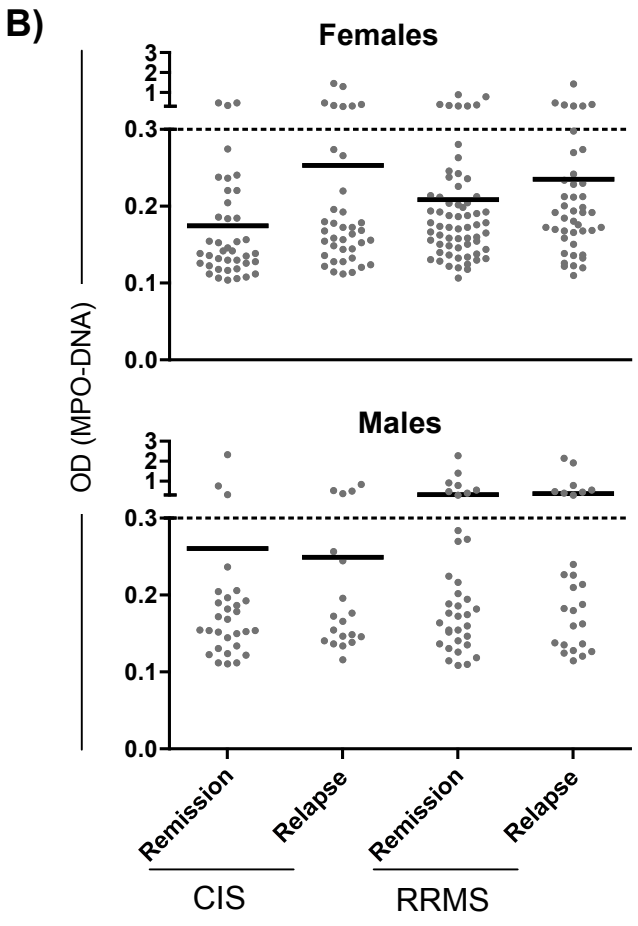

A)
SI
Females
Males
Females
Males
HC
RRMS
25 nM PMA
B)
RRMS
CIS
OD (MPO-DNA)
dsDNA (ng/ml)
**
Females
Males
C)
RRMS
**
dsDNA (ng/ml)
OD<0.2
OD>0.3
CIS
Females
Males
— OD (MPO-DNA) —

Figure 3


A)
Patient 1 - Male
Patient 5 - Male
Patient 2 - Male
Patient 6 - Male
Patient 3 - Female
Patient 7 - Female
Patient 4 - Female
Patient 8 - Female
OD 405 nm (MPO-DNA)
Months


B)
Females
Males
OD (MPO-DNA)
Remission
Relapse
Remission
Relapse
CIS
RRMS

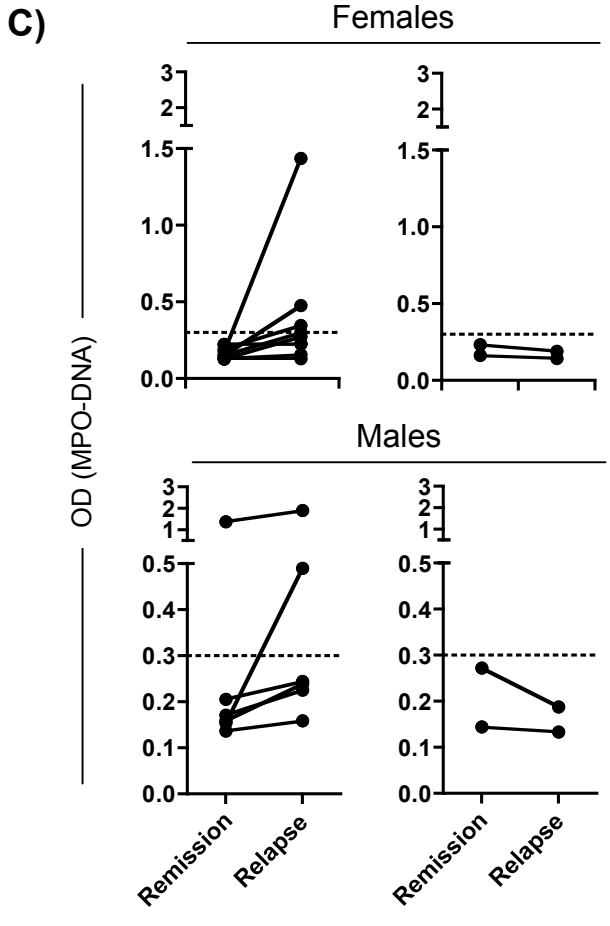

C)
Females
Males
OD (MPO-DNA)
Remission
Relapse
Remission
Relapse

Figure 4

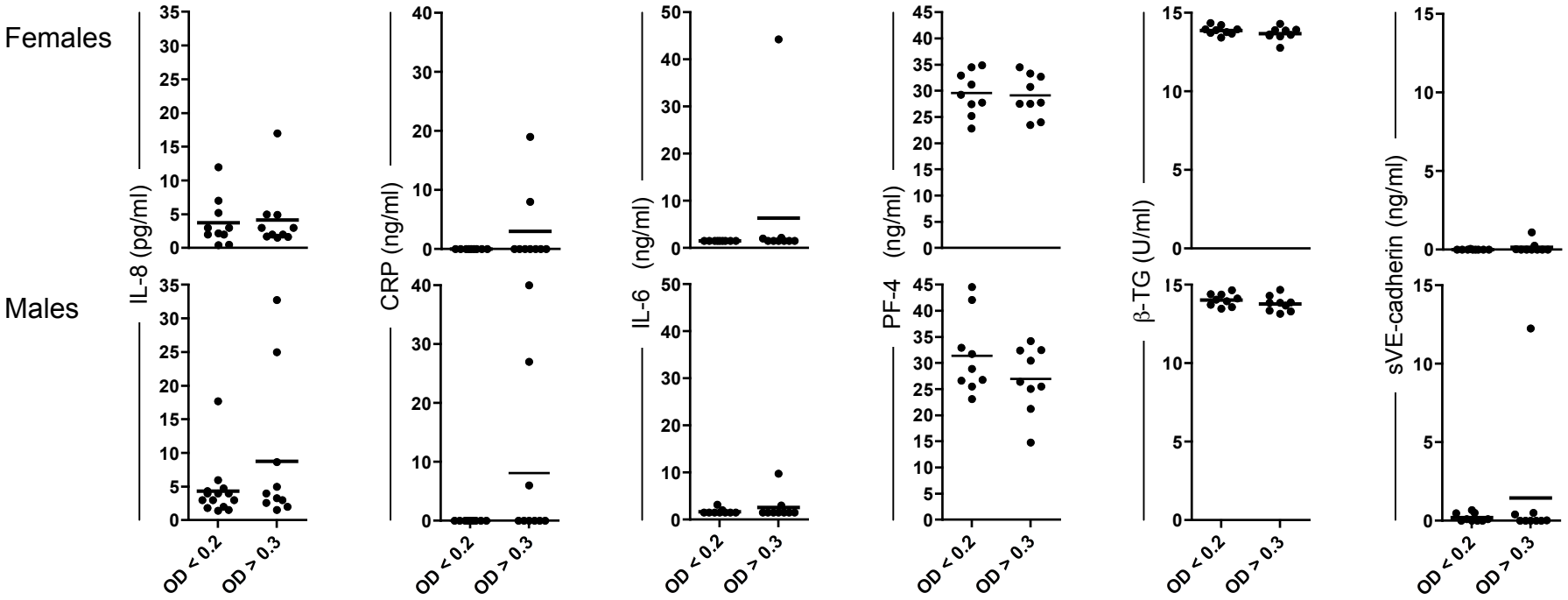

Females
Males
IL-8 (pg/ml)
CRP (ng/ml)
IL-6 (ng/ml)
PF-4 (ng/ml)
β-TG (U/ml)
sVE-cadherin (ng/ml)
OD < 0.2
OD > 0.3

Figure 5

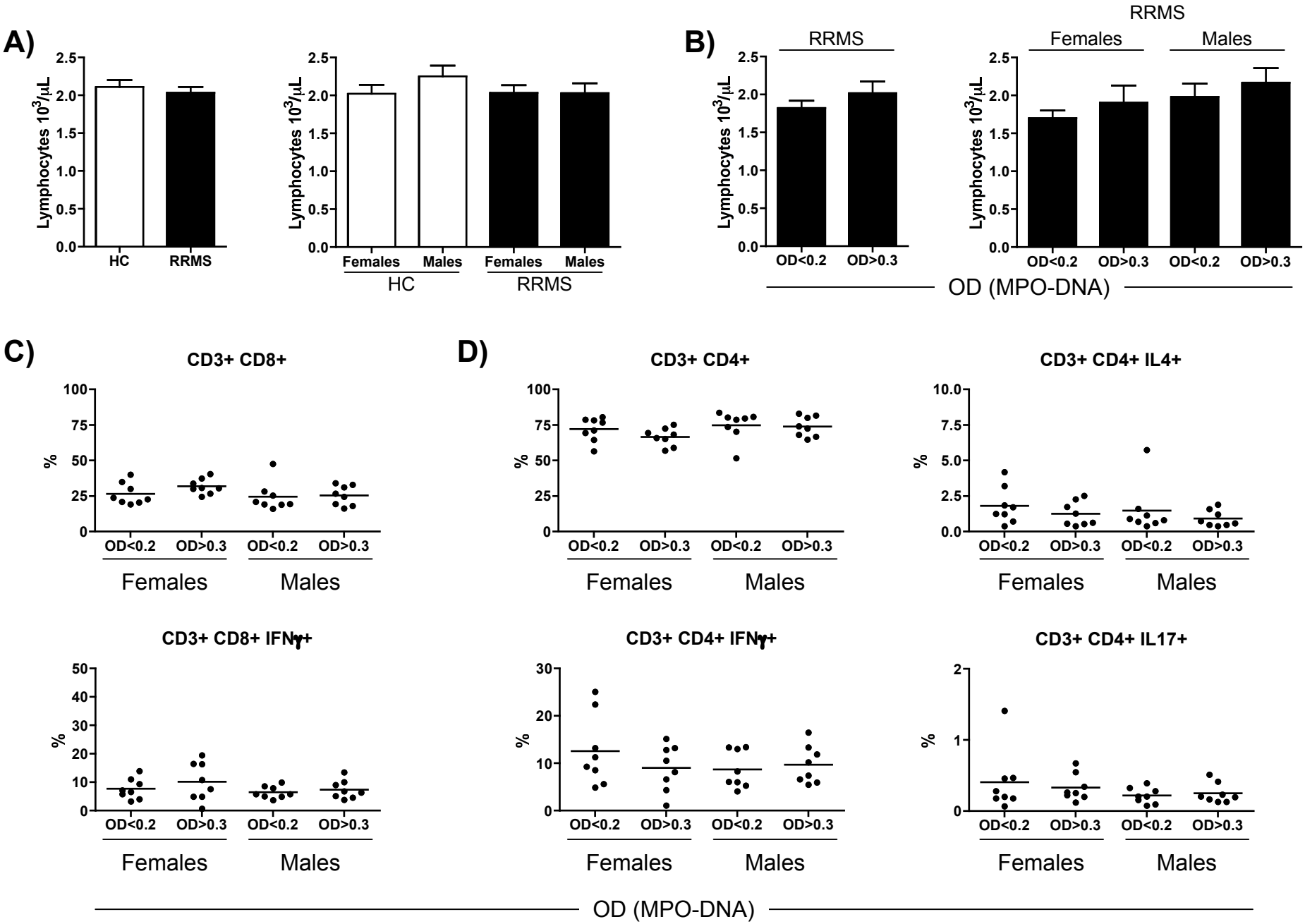

A)
Lymphocytes 10^3/µL
HC
RRMS
Females
Males
B)
RRMS
OD<0.2
OD>0.3
OD (MPO-DNA)
C)
CD3+ CD8+
%
D)
CD3+ CD4+
CD3+ CD4+ IL4+
CD3+ CD8+ IFNγ+
CD3+ CD4+ IFNγ+
CD3+ CD4+ IL17+

Figure 6

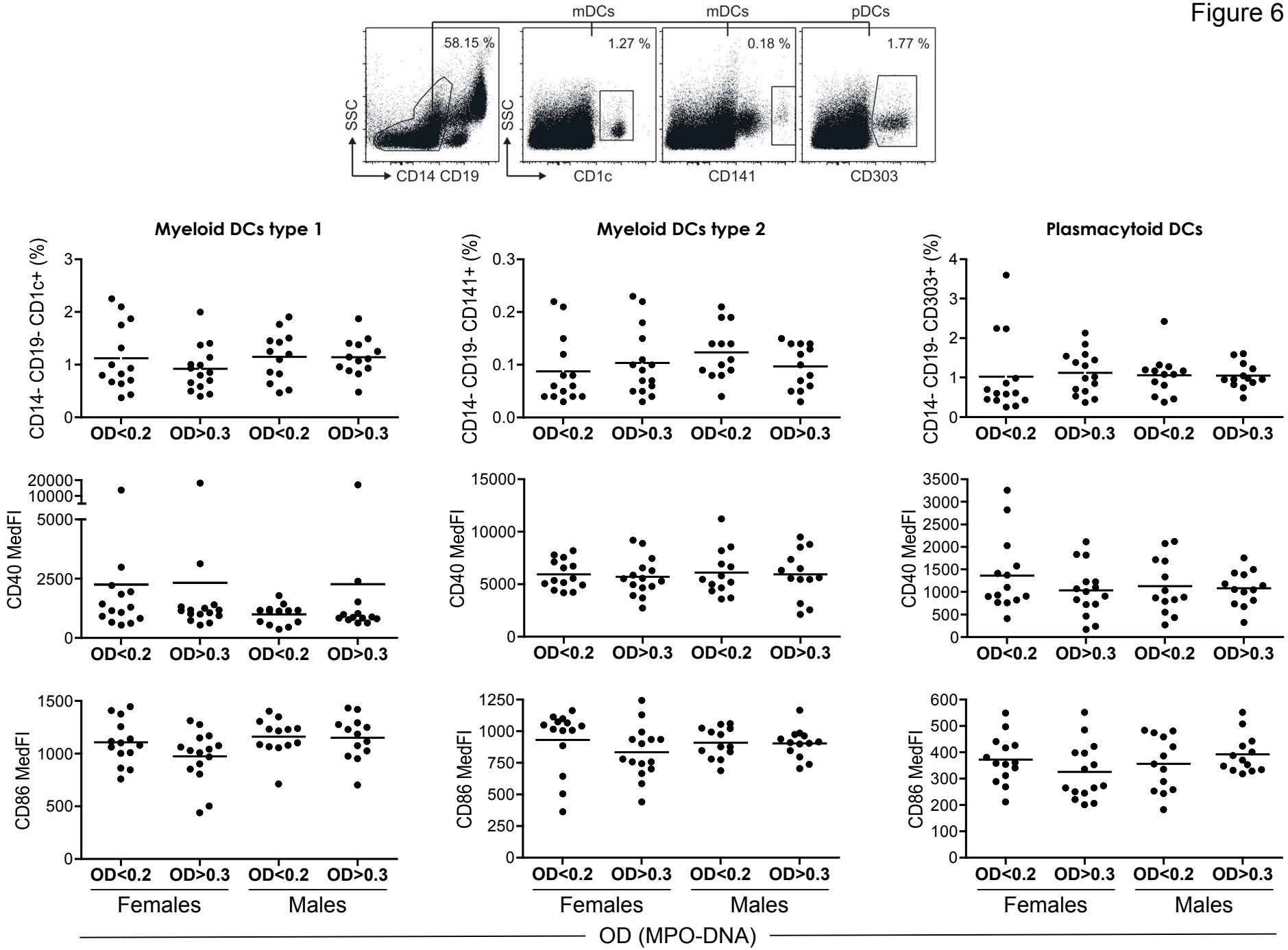
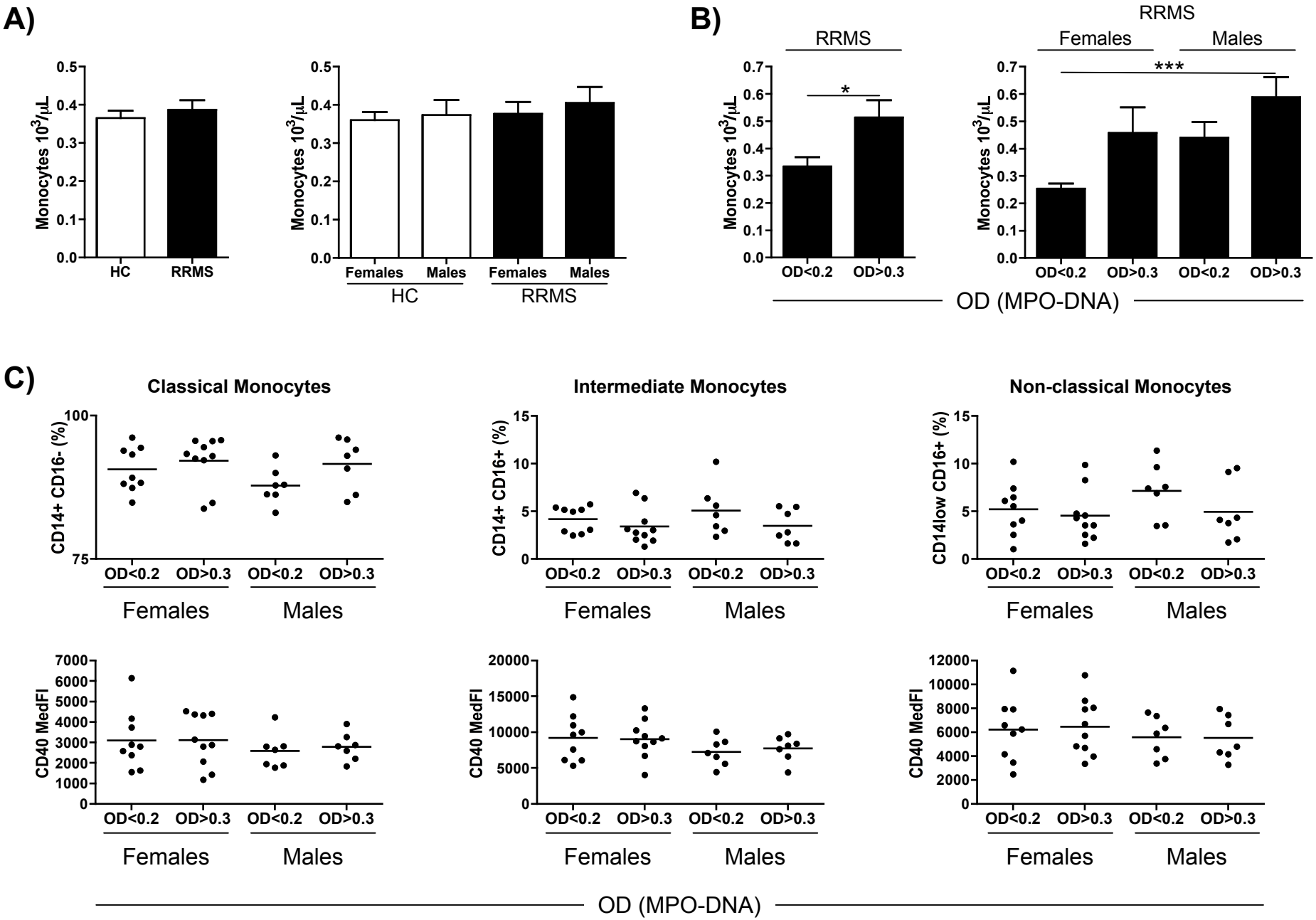

mDCs
mDCs
pDCs
58.15 %
1.27 %
0.18 %
1.77 %
SSC
SSC
CD14 CD19
CD1c
CD141
CD303
Myeloid DCs type 1
Myeloid DCs type 2
Plasmacytoid DCs
CD14- CD19- CD1c+ (%)
CD14- CD19- CD141+ (%)
CD14- CD19- CD303+ (%)
CD40 MedFI
CD86 MedFI
OD<0.2
OD>0.3
OD<0.2
OD>0.3
Females
Males
OD (MPO-DNA)

Figure 7


A)
Monocytes 10^3/µL
HC
RRMS
Females
Males
B)
RRMS
*
***
OD<0.2
OD>0.3
OD (MPO-DNA)
C)
Classical Monocytes
Intermediate Monocytes
Non-classical Monocytes
CD14+ CD16- (%)
CD14+ CD16+ (%)
CD14low CD16+ (%)
CD40 MedFI

Supplementary Table-1. Diagnose of patients with inflammatory neurological diseases other than MS (OIND) and with non-inflammatory neurological diseases (OND)

Condition	Diagnose
OIND	(Autoimmune) uveomeningeal syndrome
OIND	Idiopathic trigeminal neuralgia (left-sided)
OIND	Neuroborreliosis
OIND	Neuro-Behçet's disease
OIND	Paraneoplastic mixed axonal-demyelinating sensory-motor neuropathy (due to adenocarcinoma of the gastric cardia)
OND	Shoulder pain of unknown etiology
OND	Optical atrophy (suspected Leber's hereditary optic neuropathy)
OND	Chronic musculoskeletal pain of unknown etiology (suspected psychosomatic presentations)
OND	Muscle atrophy of unknown etiology
OND	Hypokinetic-rigid idiopathic Parkinson’s disease
OND	Tinnitus of unknown etiology
OND	Monoparesis of the right hand, most likely due to cerebral ischemia (underlying severe cerebral microangiopathy)
OND	Suspected myoclonic epilepsy with ragged red fiber (MERRF) syndrome
OND	distal paraesthesia of unknow etiology
OND	migraine with aura

Supplementary Figure 1